Protocol

Analysis of Pre-mRNA Splicing Using HeLa Cell Nuclear Extracts

Timothy W. Nilsen

This protocol is used to determine the splicing behavior of pre-mRNAs in cell extracts that are capable of carrying out splicing (e.g., nuclear extracts from HeLa cells). ³²P-labeled RNA is incubated under splicing conditions for various times, and the resulting products are analyzed on denaturing polyacrylamide gels.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Denaturing polyacrylamide gel reagents

EDTA (2 mM, pH 8.0)

Ethanol (100%)

GlycoBlue (10 µg/mL)

NaOAc (3 M, pH 5.2)

Phenol:chloroform:isoamyl alcohol (PCA; 25:24:1, w/v)

Proteinase K solution (10 mg/mL)

Radiolabeled RNA markers

RNase inhibitor (RNasin; 40 units/µL)

RNA (capped and labeled with ³²P [~15,000–20,000 cpm/ng])

RNA gel-loading buffer (1.5×) <R>

RNA splicing substrate (known to be efficiently spliced in HeLa nuclear extract)

SDS solubilization buffer (1×) <R>

Splicing extract (e.g., HeLa cell nuclear extract)

*Guidelines for the preparation of extracts are described in **Preparation of Nuclear Extracts from HeLa Cells** (Nilsen 2013). When harvesting HeLa cells, be careful to remove all the serum (which contains RNases) before the cells are disrupted. Use at least two large-volume washes before pooling the cells for homogenization.*

Adapted from *RNA: A Laboratory Manual*, by Donald C. Rio, Manuel Ares Jr, Gregory J. Hannon, and Timothy W. Nilsen. CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Splicing master mix (4×) <R>
TBE electrophoresis buffer (10×) <R>

Equipment

Dry ice
Electrophoresis equipment (including vertical gel, comb spacers, and high-voltage power supply)
Gel dryer
Heat block (set at 95°C)
Ice
Microcentrifuge
Microcentrifuge tubes (1.5-mL)
Phosphorimager equipment or X-ray film and intensifying screen
Vortex mixer
Water bath (set at 30°C)

METHOD

1. Prepare a denaturing polyacrylamide gel.
2. Set up the splicing reactions.

- i. Add the following to a 1.5-mL microcentrifuge tube:

Splicing extract	5 µL
RNasin (40 units/µL)	1 µL
³² P-Labeled RNA (~15–20,000 cpm/ng)	1 µL
Splicing master mix (4×) (make fresh for each experiment)	2.5 µL

- ii. Mix briefly by vortexing and/or flicking the tube.
 - iii. Incubate for the desired time (10–90 min) at 30°C.
3. Process the splicing reactions for polyacrylamide gel electrophoresis.
 - i. Add the following to the reaction mixture from Step 2.iii:

SDS solubilization buffer	200 µL
Proteinase K (10 mg/mL)	5 µL
 - ii. Incubate for 15 min at 42°C.
 - iii. Add 0.3 M NaOAc. Perform a PCA extraction and precipitate the RNA with ethanol containing 20 µg of GlycoBlue.

GlycoBlue acts as a coprecipitant to facilitate recovery of RNA and increase the visibility of the precipitate.
 - iv. Resuspend the RNA in 10 µL of 2 mM EDTA. Remove 2.5 µL aliquots for electrophoresis and add 5 µL of RNA-loading buffer to each sample.
4. Run a denaturing polyacrylamide gel.

- i. Set up the gel in the gel box, add running buffer (TBE) to the upper and lower reservoirs, and prerun the gel for 15–45 min at 1500 V and a maximum 45 mA.

Shorter preruns are allowed when the RNA fragments are all above 100 bases in length.
 - ii. Heat the samples (from Step 3.iv) for 1 min at 95°C and then place on ice.

FIGURE 1. An example of a splicing reaction analyzed on high (8%) and low (4%) acrylamide gels. Note the striking difference in the mobility of spliced intermediates on the two gels, particularly in the CIS lanes 10. (Reprinted, with permission, from Yu et al. 1993 [© Elsevier]).

- iii. Load the samples and run the gel at 1500 V/45 mA until the desired separation is reached for the expected products and intermediates from the reaction.
- iv. Dry the gel and expose on a phosphorimager.
5. Examine and analyze the image pattern.

Some splicing intermediates such as lariat exon 2 and other branched molecules (such as those produced by trans-splicing) will have abnormal mobility on gels. This altered mobility will be more dramatic in higher-percentage gels. It may be necessary to run more than one gel to resolve all of the products and intermediates (Fig. 1). See Troubleshooting.

TROUBLESHOOTING

Problem (Step 5): Splicing efficiency is low or nonexistent.

Solution: It may be necessary to prepare a new nuclear extract. Before asking any specific question, make sure that the extract is active. We suggest using a splicing substrate that is known to be efficiently spliced in HeLa nuclear extracts (e.g., adenovirus major late or MINX RNA). If the substrate is spliced $\geq 50\%$ in the extract, proceed with the experiment. If the substrate is spliced poorly or not at all, review the steps in the splicing protocol, and even if everything was performed correctly, a new extract must be prepared. An extract that is “dead” cannot be rescued.

Problem (Step 5): Nuclease activity is a problem (i.e., the substrate is degraded).

Solution: Make sure that the substrate is capped. In addition, ligation of unlabeled pCp to the 3′ end of the substrate can stabilize the substrate caused by the inhibition of 3′ to 5′ exonucleases.

RECIPES

RNA Gel-Loading Buffer (1.5×)

Reagent	Quantity (for 10 mL of 1.5×)	Final concentration
Formamide, ultrapure	9.5 mL	95%
Bromophenol blue (2.5%, w/v)	100 μ L	0.025%
Xylene cyanol FF (2.5%, w/v)	100 μ L	0.025%
EDTA (0.25 M, pH 8.0)	200 μ L	5 mM

Use 5 μ L for a 2.5- μ L sample. Purchase a distilled, deionized preparation of formamide and the above loading dyes. Store in small (1-mL) aliquots for up to 1 yr at -20°C . This solution is available commercially (Ambion) and is recommended over homemade.

SDS Solubilization Buffer (1×)

Reagent	Quantity (for 500 mL)	Final concentration
EDTA (500 mM, pH 8.0)	1 mL	1 mM
SDS (sodium dodecyl sulfate; 10%)	25 mL	0.5%
Tris-Cl (1 M, pH 7.5)	10 mL	20 mM
H ₂ O, RNase-free	464 mL	–

Store for up to 1 yr at room temperature.

Splicing Master Mix (4×)

Reagent	Quantity (for 62.2 µL)	Final concentration (1× in the splicing reaction)
Creatine phosphate (0.5 M)	10 µL	20 mM
ATP (100 mM)	5 µL	2 mM
KCl (3 M)	2.5 µL	30 mM*
MgCl ₂ (0.5 M)	2.1 µL	4.2 mM
Dithiothreitol (DTT) (1 M)	1 µL	4 mM
Tris (1 M, pH 8.0)	1 µL	16 mM
Aprotinin and leupeptin stock solution**	1 µL	
Polyethyleneglycol (PEG 8000; 20%, w/v)***	37.5 µL	3%
H ₂ O	2.1 µL	

Prepare fresh and keep on ice.

*The final KCl concentration in the reaction mixture is usually between 50 and 80 mM because the splicing extract also contains KCl, which contributes to the final concentration. Adjust according to the concentration in dialysis buffer and the amount of extract used in the reaction.

**Prepare the aprotinin and leupeptin stock solution immediately before use by adding 1 µL of a 10-mg/mL solution of aprotinin and 1 µL of a 2-mg/mL solution of leupeptin to 38 µL of H₂O.

***Weigh out 8 g of PEG 8000 (e.g., Carbowax PEG 8000 from Fisher) in a 50-mL conical tube. Add RNase-free H₂O to the 40-mL mark on the tube. Close the lid tightly and heat briefly at 65°C to dissolve. Store for 4 mo at room temperature.

TBE Electrophoresis Buffer (10×)

Reagent	Quantity (for 1 L)	Final concentration
Tris base	121.1 g	1 M
Boric acid	61.8 g	1 M
EDTA (disodium salt)	7.4 g	0.02 M

Prepare with RNase-free H₂O. Dilute 100 mL to 1 L to make gel running buffer. Store for up to 6 mo at room temperature.

REFERENCES

- Nilsen TW. 2013. Preparation of nuclear extracts from HeLa cells. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot075176.
- Yu Y-T, Maroney P, Nilsen TW. 1993. Functional reconstitution of U6 snRNA in nematode *cis*- and *trans*-splicing: U6 can serve as both a branch acceptor and a 5' exon. *Cell* 75: 1049–1059.

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